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INNOVATIVE RATION PRESERVATION VIA SUPERCRITICAL CARBON DIOXIDE

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Final Report
October 2006 – June 2010

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14. ABSTRACT

This report describes a 4-year effort, ended June 2010, to study the effectiveness of supercritical carbon dioxide ($SCCO_2$) processing in destroying unwanted enzymes and microbes before spoilage, browning, or other damage to the food can occur. $SCCO_2$ is a non-thermal, generally regarded as safe (GRAS) treatment and a novel alternative to traditional thermal processing, which can cause product quality degradation. The CO_2 is pumped into a holding tank where the pressure and temperature has been manipulated to bring it to a $SCCO_2$ state (above 1071 psi and 31.1C), at which point it is introduced to the food item. The results of this research, conducted by the Natick Soldier Research, Development and Engineering Center (NSRDEC), Combat Feeding Directorate (CFD), show that $SCCO_2$ inactivates enzymes and microbes without subjecting foods to damaging side effects. Enzymatic assays, spectrophotometry, microbial assays, fluorescence, and sensory analysis were performed throughout the study. The report includes a discussion of the direction of future research related to $SCCO_2$ processing.

15. SUBJECT TERMS

FOOD	FOOD QUALITY	MILITARY RATIONS	NONTHERMAL PROCESS
SCCO2	FOOD SPOILAGE	FOOD PRESERVATION	MICROBIAL INACTIVATION
RATIONS	CARBON DIOXIDE	MICROBIAL CONTROL	ENZYMATIC INACTIVATION
QUALITY	MICROORGANISMS	SUPERCRITICAL FLUIDS	POLYPHENOL OXIDASE(PPO)
FOOD SAFETY	FOOD PROCESSING		

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Preface

This report documents the progress of a Science and Technology initiative for a new food processing technology that uses supercritical carbon dioxide (SCCO2). The Natick Soldier Research, Development and Engineering Center (NSRDEC), Combat Feeding Directorate (CFD) conducted this work under the project entitled, "Innovative Ration Preservation via Supercritical Carbon Dioxide", TB 07-04, funded by the Combat Feeding Research and Engineering Program from October 2006 through June 2010. The data and accomplishments from this project were transitioned to a JSN in FY11 for further development and application to ration items.

This report details the initial research that was performed on model systems to determine the effectiveness of enzymatic and microbial inactivation. The SCCO2 processing was then applied to small food samples for further development and optimization. Most of the processing was completed on instruments procured from Supercritical Fluids Technologies (Newark, DE) model name SFT100. During the course of the study, microbial and enzymatic assays were conducted after processing. Select sensory attributes were examined, but due to the location of the processing system in a chemical wet lab, taste could not be evaluated. At the end of the study, a more robust instrument was used for all processing. This instrument was located at NovaSterilis (Ithaca, NY), model name Nova 2200.

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INNOVATIVE RATION PRESERVATION VIA SUPERCRITICAL CARBON DIOXIDE

1.0 Introduction

Novel treatments to improve food safety and quality have always been a focus for combat rations. There are many unique challenges that exist when it comes to providing the Warfighter with healthy, safe, stable, and palatable food in the field. Microbiological, enzymatic, and physiochemical reactions continuously occur in food, causing constant changes. Food enzymes and microbes are associated with loss of product quality such as browning, off-flavors, and odors. Additionally, a required 3-year shelf life can give rise to browning and other reactions that degrade quality of food and promote unwanted microbial growth (e.g., thermophillic bacteria), especially in adverse environments. Traditional thermal processing of some ration components can also cause product quality degradation. There is a critical need in combat rations for a generally regarded as safe (GRAS), non-thermal processing treatment that will inactivate undesirable enzymes and microbes before quality degradation and/or food safety issues can occur.

This report documents the progress of an initiative for a novel food processing technology that uses supercritical (SC) carbon dioxide (CO_2) as a GRAS alternative to traditional thermal processing of some ration components. The Natick Soldier Research, Development and Engineering Center (NSRDEC), Combat Feeding Directorate (CFD) conducted this work from October 2006 to June 2010.

1.1 Background

An SC fluid is a fluid that has been brought to a state above its critical point by increasing its temperature and pressure above its standard temperature and pressure. At this critical point, the SC fluid has the properties of both a liquid and gas, and a clear distinction between the two is not discernable. This supercritical state is imperative to the functionality of this process because the fluid offers the increased diffusivity of a gas and the solvency of a liquid. As a result, SC fluids have been used in applications that range from decaffeinating coffee to the sterilization of medical instruments.

CO_2 is generally the chosen fluid for SC processing for a number of reasons. It has a relatively low SC temperature and pressure (above 31.1°C and 1072 psi, respectively) and has negligible impact on the environment. SCCO2 is non-toxic and nonflammable, has GRAS status, and naturally occurs in many foods.

SCCO2 is being studied as a potential and desirable food processing method primarily because it does not involve the use of high temperatures. Thermal processing technologies can negatively affect a food's organoleptic and nutrient properties. SCCO2 has been found to inactivate enzymes and microbes without the detrimental thermal effects. Historically, this research has been limited to liquid model systems and liquid foods, including enzymes such as α-amylase in a model system (Yoshirma et al. 2001) and Polyphenoloxidase (PPO) extracted from spiny lobster

(Chen et al. 1992). Microbes such as *Escherichia coli* (*E. coli*) *K12* in apple cider (Yuk et al. 2010), *Lactobacillus brevis* (*L.brevis*), and *Saccharomyces cerevisiae* (*S. cerevisiae*) in model systems (Shimoda et al., 1998) have also been inactivated. Limited work has been done with studying the effects of SCCO2 on semi-solid or solid foods.

1.2 Tasks

The major tasks for this study included:

- Procure a manufacturer's system for SCCO2 processing
- Determine a model system to be used for initial studies to explore delivery and treatment parameters
- Optimize the SCCO2 processing parameters, including temperature, pressure, soaking time, and chamber de-pressurization
- Develop microbial and enzymatic protocols and assays for continued measurement
- Determine the penetration levels of SCCO2 in a semi-solid model system
- Increase microbial studies to include pathogens
- Employ processing on particle food items
- Analyze the results

2.0 Experimental Approach

In 2006, NSRDEC, CFD began a science and technology research initiative employing the SCCO2 batch processing method to semi-solid and solid foods. Most of the processing performed during this project was completed using the SFT100 batch system procured from Supercritical Fluids, Inc (Newark, DE), shown in Figure 1. This system was manufactured to be used as an extractor, and thus it does not have the necessary precision needed for food processing, requires manual operation, and has limited capacity. The SFT100 holding vessel accommodates only up to 100 ml. Therefore, only very small batches can be processed at a time, i.e., cubes of vegetables, fruits, or meats. The investigators chose the SFT100 due to its price point (approximately $27,000 in 2006) and because manufacturers were not manufacturing and/or marketing instrumentation for food processing. The research using the SFT100 is discussed in detail in Chapter 3.

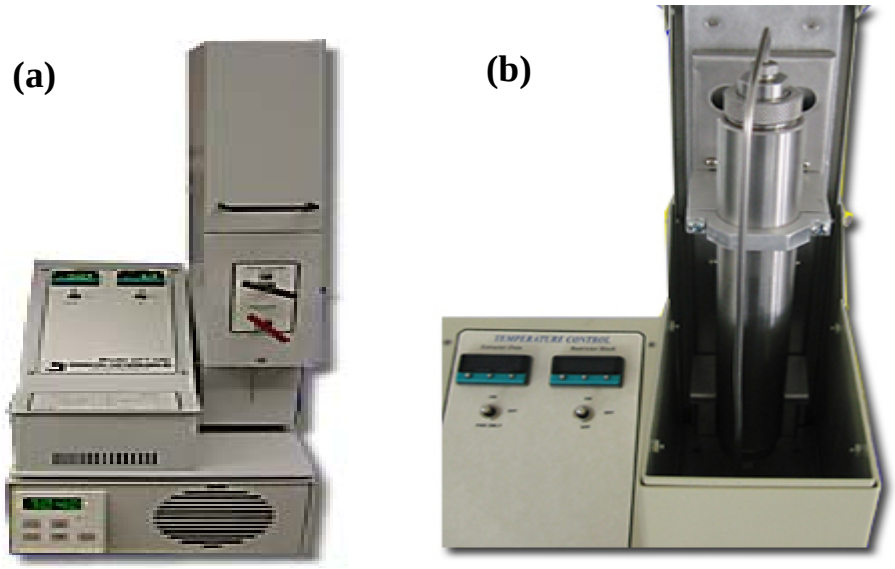

Figure 1: The SFT100 Processing Instrument. (a) Exterior; (b) Interior Holding Vessel.

The initial research using the SFT100 was performed on model systems or basic foods to determine the effectiveness for enzymatic and microbial inactivation. Following success on microbial model systems, microbial inactivation was tested on more complicated food matrices for further development and optimization. Those tests were successful, but a more robust instrument was needed for further research due to the SFT100's manual operation and limited capacity.

Given that instrumentation for food processing was still not being manufactured and/or marketed, novel instrumentation was sought. NovaSterilis, a small company in Ithaca, NY, was manufacturing an instrument (Nova 2200) for sterilizing biological tissues such as bone and skin. The Nova 2200 can hold up to 20 L of sample and is completely computerized. This instrument is used for and was designed for SCCO2 processing of delicate materials such as live dermal tissue, similar in nature to the delicate tissue of fresh fruit. Thus, it was believed that it was likely to succeed for food processing. Selected food items were transported to NovaSterilis and

were processed using the Nova 2200. The samples were then transported back to NSRDEC for enzymatic and microbial testing. The processing and testing are discussed in Chapter 4. Neither the research using the SFT100 nor the Nova 2200 completely elucidated the SCCO2 processing mechanism, and other studies have shown there may be a number of mechanisms involved. Thus, research with diffusivity studies, phenolic activity, and confocal laser scanning microscopy was initiated near the end of this project. Previous studies of the SCCO2 processing mechanism and the efforts during this project are discussed in Chapter 5.

3.0 SFT100 Processing and Testing

Enzymatic (PPO) and microbial samples, of both fresh fruits and vegetables and model systems (enzymes or microbes in buffer solutions) were treated with SCCO2 using the SFT100 processing instrument. Each sample item was prepared and placed in a 50-ml polypropylene conical tube and then placed in the holding vessel of the instrument. The holding vessel was then sealed with a large pressure nut (see Figure 1b) and clamped down. The liquid CO_2 was opened at the tank and pumped into the instrument. The CO_2 entered the chamber where the temperature and pressure had been elevated to bring it to its supercritical state. The SCCO2 was then introduced to the food in the chamber and allowed to soak for a predetermined time. Once the food had soaked in the SCCO2, the vessel was slowly depressurized, and the samples were removed from the system.

3.1 PPO Enzymatic Inactivation

Initial enzymatic work was performed using purchased mushroom tyrosinase (Sigma-Aldrich, Missouri) in an aqueous solution. The tyrosinase model system presented a 60% inactivation rate at processing parameters of 3500 psi, 42˚C, and 45 min soaking. Due to the high cost of tyrosinase, which is also a PPO, enzymes extracted from fruits and vegetables were used for the remaining processing parameter research. Pieces of potatoes, apples, and lettuce were processed with SCCO2 in the SFT100, and then the PPO was extracted from the samples for enzymatic assays.

3.1.1 Sample Processing

The tube containing each food sample was placed into the instrument vessel and then processed. The samples were processed in a range of 1000-4350 psi, with a soak time of 1-120 min and a temperature of approximately 40°C (±1-3˚C). It was discovered that the 4350 psi/20 min soaking time had the highest rate of inactivation of >99%, significantly greater than the 63% inactivation rate found at 3500 psi. Control samples were held at ambient temperatures during the processing time and presented high amounts of PPO activity.

3.1.2 Assay Procedure

Regardless of exposure time or sample size, the assay used to determine the level of PPO activity was consistent for all samples. The enzyme extraction buffer contained 0.5 M sodium phosphate and 0.1 M sodium fluoride adjusted to a pH of 7.0 with 0.1 N of sodium hydroxide. A ratio of 10 to 1 extraction buffer (ml) to fresh tissue (g) was used for the extraction. The sample and buffer were homogenized on high speed for 20 s using a hand-held tissue miser, which was cleaned with distilled water between each sample. The homogenate slurries were centrifuged at 10,000 rpm at 5°C for 30 s to remove large insoluble particles. The cuvettes for the PPO assay were prepared with 800 µL of assay buffer (0.5 M sodium phosphate/0.1 M Sodium Fluoride) and 100 µL of enzyme extract and mixed thoroughly. Once the reaction started, 100 µL of catechol, which served as the enzyme substrate, was added to the mixture. Reactions were read at 420 nm for 1 min on a spectrophotometer at the greatest change of rate.

3.1.3 Assay Results

The PPO activity in the centrifuge tubes and cuvettes was evident with the naked eye (Figures 2 and 3, respectively). In Figure 2 the control sample (Co) is visibly darker than the samples (PPO extracted from lettuce) processed for 30 min and 45 min (i.e., Samples 30m and 45m, respectively) before the catechol was added. In Figure 3, the cuvette processed for 45 min at 3000 psi with catechol (substrate) added is visibly lighter than the control sample with catechol added, due to enzymatic inactivation in the processed cuvette.

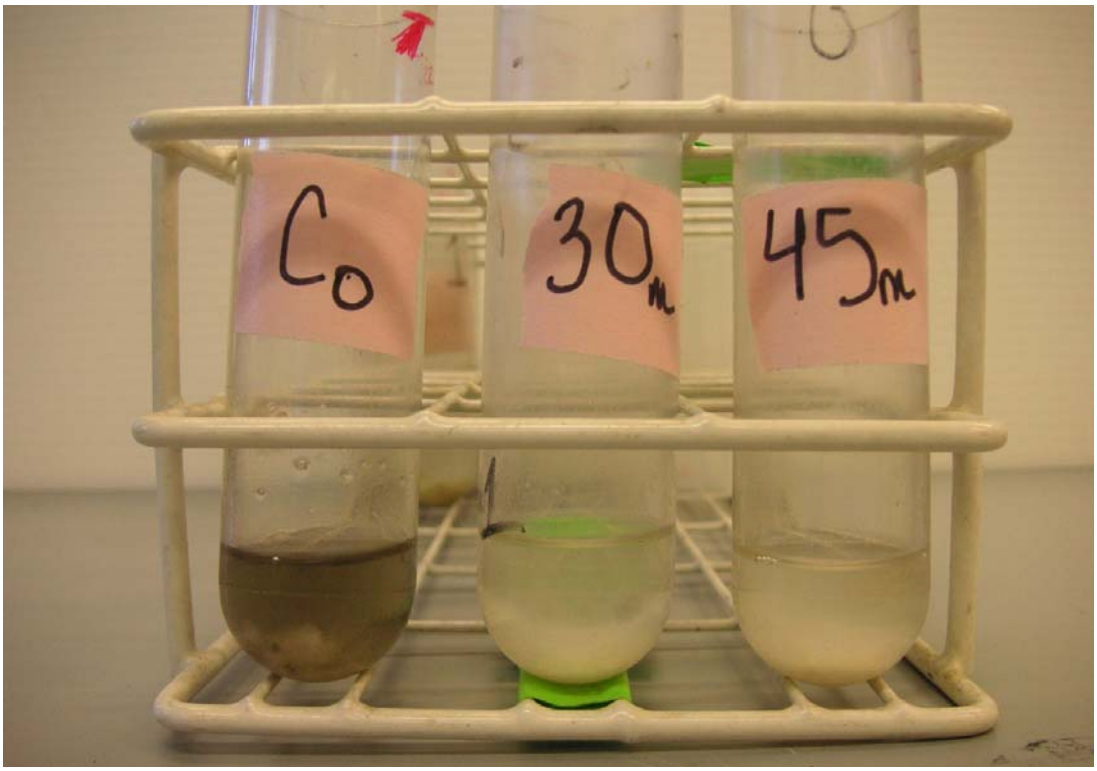

Figure 2: Enzymatic Inactivation Test Tubes before Catechol Was Added.
Left: Unprocessed PPO Extracted from Lettuce;
Center: PPO extracted from Lettuce Processed for 30 min at 4350 psi;
Right: PPO Extracted from Lettuce Processed for 45 min at 4350 psi.

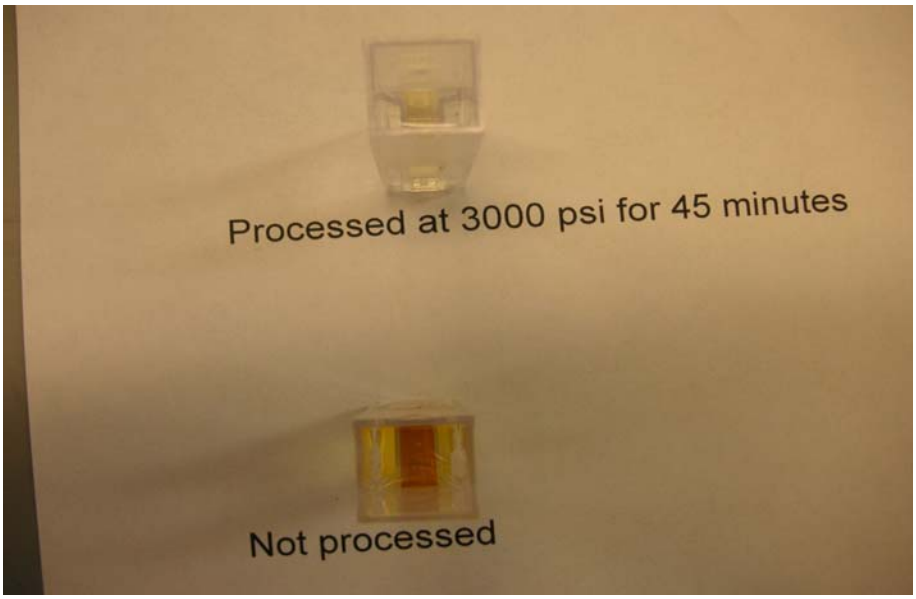

**Figure 3: Enzymatic Assay Cuvettes with Catechol Added.
Top: PPO Extracted from Lettuce Processed for 45 min at 3000 psi; Bottom: PPO Extracted from Unprocessed Lettuce.**

The percent of enzymatic (PPO) inactivation (based on the activity of the enzyme in the control sample compared to the processed sample) achieved at the various temperatures, pressures, soaking times, and exhaust times is listed in Appendix A.

Cubed potatoes were also processed as described in Section 3.1.1. They were then placed in 4°C storage in conical tubes, along with control potatoes in conical tubes, for 27 days. Enzyme assays were performed during that 27-day storage study. Figure 4 shows the enzymatic activity of three potato samples that were processed at 4350 psi for 60 min compared with the activity of the control sample. The control potatoes retained their enzymatic activity for the duration of the storage study, and the processed potatoes did not present enzymatic activity. The browning of the potatoes was monitored over a 48-h period at the beginning of the storage study. The processed potatoes remained white after processing, and the control potatoes began to turn a grayish-brown color immediately upon cutting (Figure 5). The image in Figure 5 was taken 12 h after the samples were processed at 4350 psi for 45 min. The potatoes were then placed under an ultraviolet (UV) light (350 nm) to confirm the occurrence of enzymatic browning via the quenching of fluorescence. Since enzymatic activity quenches fluorescence, this relationship can be used to quickly determine the inactivation of enzymes. In Figure 6 the lack of fluorescence in the control (unprocessed) potatoes confirms that a very high amount of enzymatic (PPO) activity took place. The fluorescence in the two samples of SCCO2 processed potatoes indicates inactivation of PPO. The different levels of fluorescence in two samples indicate a greater amount of inactivation in the sample treated at 4350 psi than the one treated at 1150 psi.

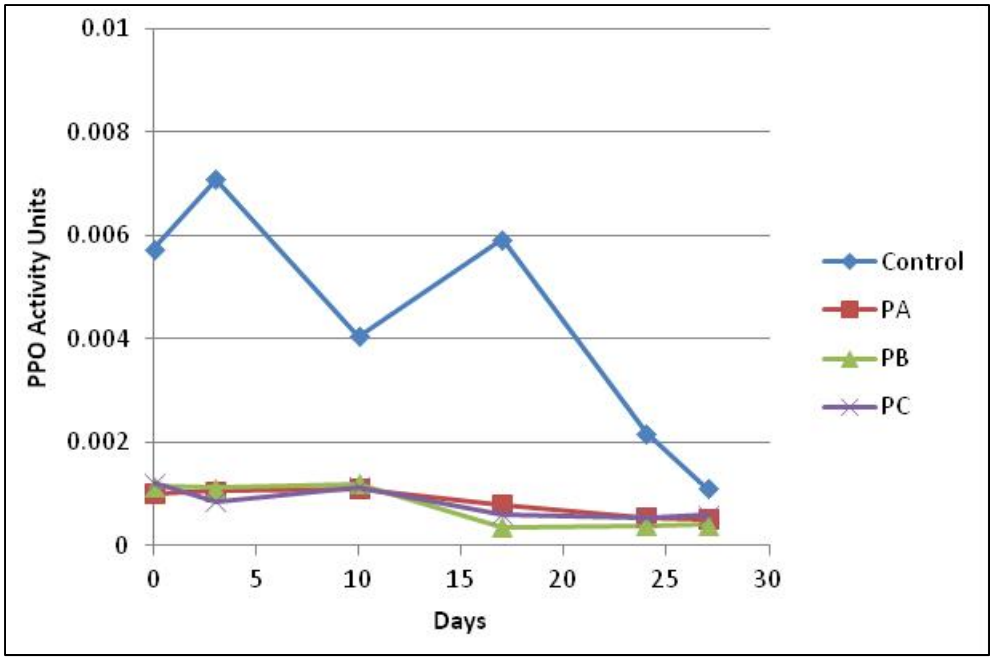

Figure 4: Enzymatic Activity of Potato Samples Processed at 4350 psi for 60 min and Stored for 27 Days.

Figure 5: Unprocessed Potatoes (Left) and Potatoes 12 h after Processing at 4350 psi for 45 min (Right).

Figure 6: UV Images of Potatoes 12 h after Processing.
Top: Processed at 4350 psi for 45 min;
Middle: Processed at 1150 psi for 45 min;
Bottom: Unprocessed.

Lettuce and avocado samples were also treated as described in Section 3.1.1 at 4350 psi/42˚C for various lengths of treatment times with similar results. Figure 7 shows that the avocado samples treated with SCCO2 for 60 min (right) were much greener than the untreated avocado samples (left) after 72 h in storage at ambient conditions. Both samples were administered lemon juice immediately after cutting.

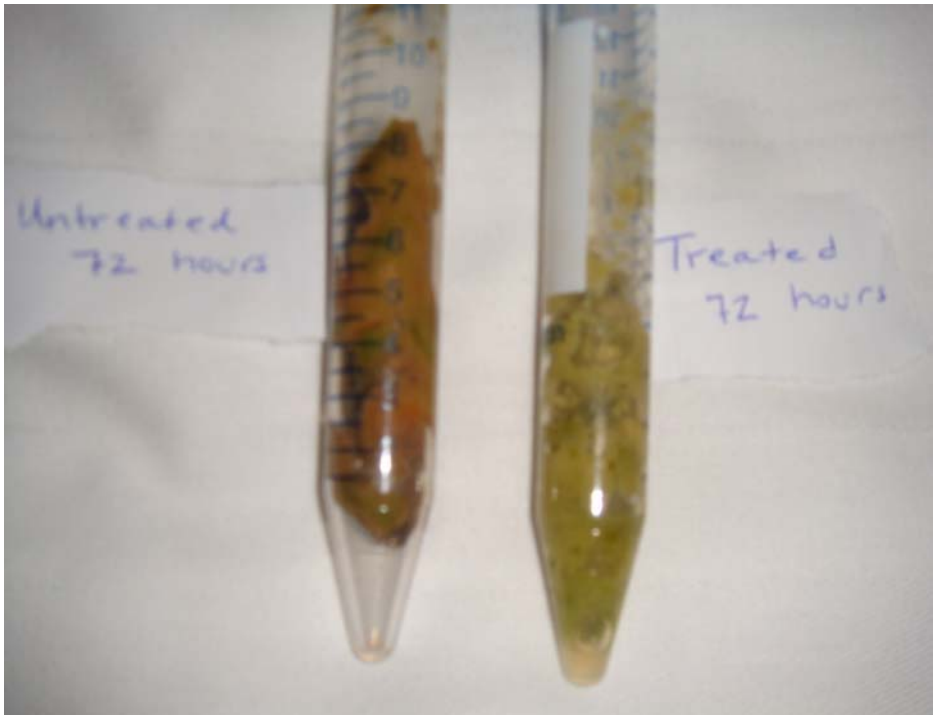

Figure 7: Unprocessed Avocadoes (Left) and Avocadoes 72 h after Processing at 4350 psi for 60 min (Right).

3.2 Microbial Inactivation

Microbial inactivation was successfully performed on model systems initially for proof of concept (successful results are noted in Section 3.2.1). Following the success on model systems, microbial inactivation was tested on more complicated food matrices. The non-pathogenic microbes were tested prior to the pathogenic for method development. It was found that a lower pressure was needed for microbial inactivation when compared to enzymatic activation.

3.2.1 Model Systems

The SCCO2 was initially administered to a model system of phosphate buffered saline (PBS). Two ml of buffer was inoculated with 10^7 cfu/ml of *L. reuteri* and then processed. A 1-ml sample was taken from each treatment and from the control and was serial diluted (1 to 10) in PBS, and 100 µL was spread plated onto *L.* MRS agar (Becton, Dickinson Company, Sparks, MD). The petri plates were incubated at 37°C anaerobically for 48 h before enumeration. The results can be found in Table 1. Additional information (i.e., the processing temperatures, exhaust times, and microbial log reductions) is listed in Appendix A.

Table 1: Results of SCCO2 Processing of Non-Pathogenic Microbial Model Systems.

Pressure (psi)	Treatment time (min)	cfu/ml
Control	0	2.0×10^7
1070	30	8.3×10^6
1070	60	5.05×10^4
1070	120	$<10^1$
3620	30	6.15×10^6
3620	60	$<10^1$
3620	120	$<10^1$
4350	30	7.65×10^5
4350	60	$<10^1$
4350	120	$<10^1$

3.2.2 Non-Pathogenic Inactivation

Cubes of beef, sized to fit into 15 ml Falcon tubes, were inoculated by dipping them into a prepared culture of *E. coli* ATCC 15977 at 10^6 cfu/ml (1 to 1000 dilution) in PBS 0.3% Tween® 20. Each beef cube was soaked for 20 s in the inoculation and then transferred to a pre-weighed weigh boat, where it was allowed to air dry for 15 to 20 min. The total weight was determined, and the difference was recorded per treatment. The inoculated tubes were transferred to the Falcon tubes. After treatment, each meat cube was transferred to a sterile stomacher bag with 10 ml of PBS 0.3% Tween® 20 and homogenized in the stomacher for 2 min. The homogenate was sampled, and 100 µL was plated onto a plate count agar and incubated at 27°C for 24 h before colony enumeration. Dilutions plated were 10^{-1} to 10^{-6}. The results can be found in Table 2. Additional information, (i.e., the processing temperatures, exhaust times, and microbial log reductions) is presented in Appendix A.

Table 2: Results of SCCO2 Processing of Non-Pathogenic Beef Inoculated with *E. coli* 15977.

Sample	Pressure (psi)	Treatment Time (min)	Log cfu/g
A	4350	30	2.89
B	Control	0	4.39
C	4350	60	1.8
D	Control	0	4.39
E	1060	60	3.56
F	Control	0	4.36

3.2.3 Pathogenic Inactivation

The same procedure used for the non-pathogenic beef study was employed for the pathogenic beef study. Overnight growth culture of *E. coli* 0157:H7 ATCC 43888 was diluted in PBS at 2 ml into 35 ml PBS and vortexed. It was dip inoculated for 2 min and refrigerated at 4°C until processing. After SCCO2 processing (42°C and 4350 psi), samples were pulsified for 30 s and hand massaged in Butterfield's phosphate buffer then spread plated on MacConkey sorbitol agar and incubated aerobically for 18 h for microbiological analysis. The results are in Figure 8. Additional information, (i.e., the processing temperatures, exhaust times, and microbial log reductions) and the results of the three samples processed at 38°C and 4350 psi are listed in Appendix A. Microbial images of both the unprocessed beef and the beef processed at 42°C and 4350 psi for 60 min are shown in Figure 9. The microbes in the unprocessed sample (right side) are too numerous to count.

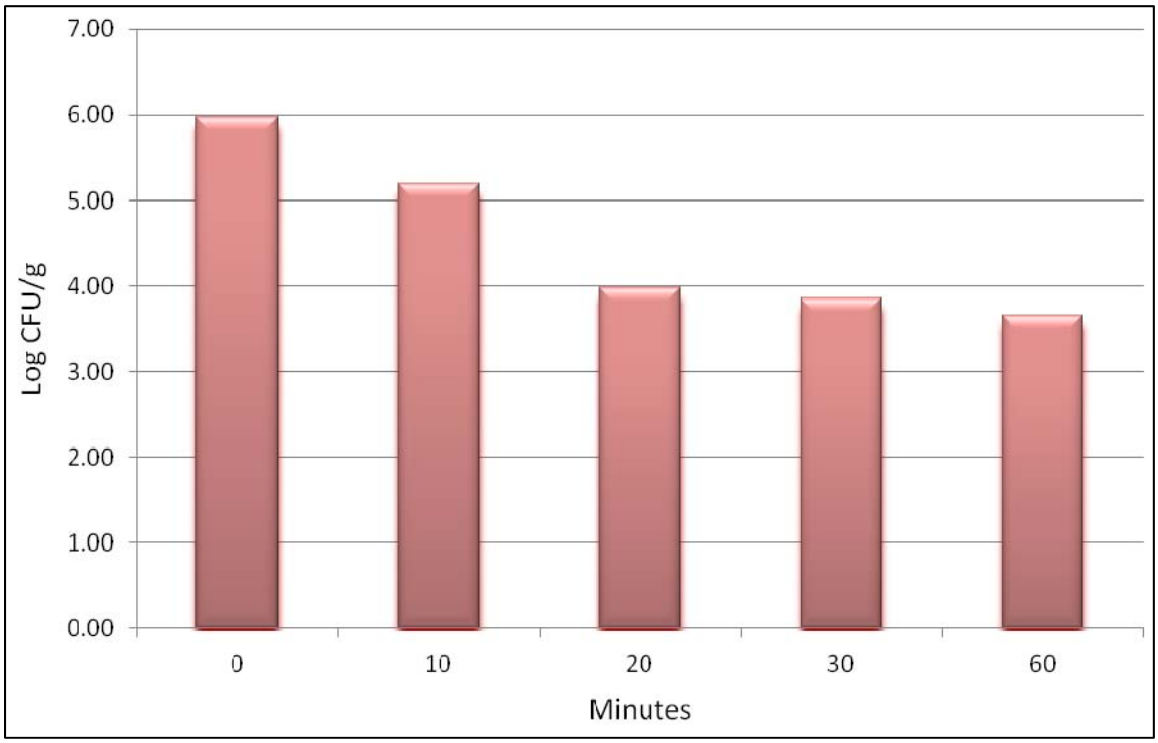

Figure 8: Beef Inoculated with *E. Coli* 0157:H7 and Processed at 42°C and 4350 psi for 60 min.

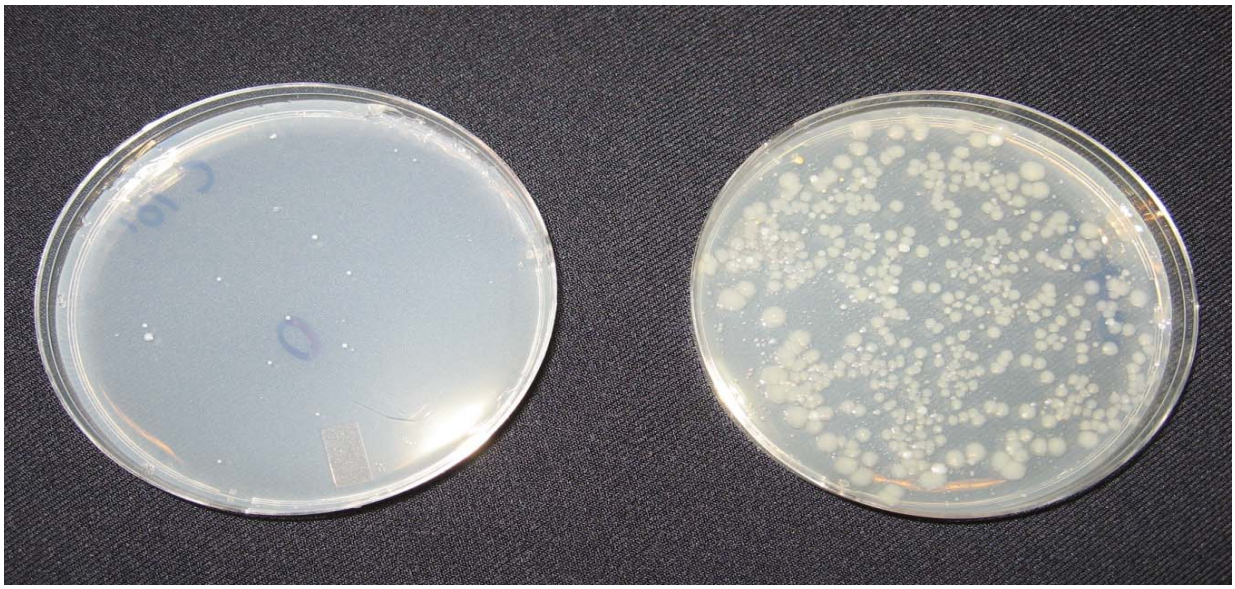

Figure 9: Microbial Plates of Beef Inoculated with *E. Coli* 0157.
Left: Processed at 42°C and 4350 psi for 60 min; Right: Unprocessed.

4.0 Nova 2200 Processing and Testing

4.1 Nova 2200 Processing

The NovaSterilis instrument utilizes a food grade acid (accelerant) that is added to the processing chamber prior to operation. This accelerant is a processing aid which expedites the sterilization processing times. Mechanisms for explaining this benefit are yet to be determined but are likely a result of increased diffusivity of the acid in the supercritical state which enables it to penetrate bacterial membranes and/or denature protein structures more readily. This particular run utilized lactic acid (LA) as the accelerant due to its GRAS status and its extensive use throughout the commercial food industry and the meat industry in particular.

4.1.1 Processing Parameters and Methodology

Selections of salami, pastrami, summer sausage, mashed potatoes, and applesauce were transported from NSRDEC to NovaSterilis for processing on the Nova 2200. The parameters for processing were: 10-min and 30-min treatment times, 1436 psi, and a maximum temperature of 35°C in the internal chamber.

Prior to processing, items were packaged into sterile Tyvek pouches which allow the SCCO2 to flow easily through its semi-permeable barrier layer while providing adequate protection from environmental contamination. Following SCCO2 processing, the processed samples and the controls were both overwrapped in trilaminate foil pouches with an oxygen scavenger sachet and then hermetically sealed. All food variables were identical with the exception of the SCCO2 treatment times (0, 10, and 30 min). The specifications for the preparation of each sample item can be seen in Table 3.

Table 3: Sample Items Processed on the Nova 2200

Sample	Preparation
Summer sausage	Whole, 1/4-in slices, 1/8-in slices, 1/16-in slices in Tyvek pouch
Pastrami	Whole, 1/4-in slices, 1/8-in slices, 1/16 inch slices in Tyvek pouch
Hard salami	Whole, 1/4-in slices, 1/8-in slices, 1/16-in slices in Tyvek pouch
Applesauce	Full package transferred to Tyvek pouch
Mashed potatoes	Full package transferred to Tyvek pouch

4.1.2 Processing Outcomes

Post-processing, an informal sensory panel was performed on all the variables to determine differences in the organoleptic properties on the treated samples versus the control or unprocessed samples. The panelists included three members from CFD, four employees from NovaSterilis, and one contractor from RDI Foods, Inc. It was concluded subjectively by the panel that there was almost no difference in the taste, texture, or odor between the processed and unprocessed samples.

There were some minor negative processing outcomes that resulted from the SCCO2 procedure that were determined to be inconsequential but worth noting. The applesauce's Tyvex pouches were initially swollen due to residual SCCO2 that was trapped in the pouches, but dissipated completely after 45 min. Additionally, the sausage and salami samples had some fat that had exuded from the meat matrices and collected in the pouch. However, the extracted fat did not affect the texture or taste of the meats. The samples were transported back to NSRDEC and placed in 49°C (120°F) storage for follow-on microbial testing.

Figures 10, 11, and 12 demonstrate the visual effects of processing on hard salami. Figure 10 shows 1/4-in slices before processing. Figure 11 compares the appearance of an unprocessed 1/4-in slice with one that was processed for 30 min. Figure 12 shows some casing separation in a whole hard salami as a result of 30 min of processing.

Figure 10: Slices of 1/4-in Hard Salami in Tyvek Pouch Prior to Processing

Figure 11: Unprocessed 1/4-in Slice of Hard Salami (left) and 1/4-in Slice Processed using Nova 2200 for 30 min at 1436 psi and 35°C (right).

Figure 12: Whole Hard Salami after Nova 2200 Processing for 30 min at 1436 psi and 35°C.

4.2 Nova 2200 Microbiological Analysis

4.2.1 Storage and Analysis Methodology

The samples were transported back to NSRDEC in a cooler to prevent outgrowth of any surviving bacteria or yeast. Sterility testing was conducted following a pre-enrichment of the processed samples by incubation at 35°C for 10 days. Microbial testing was done immediately on control and processed samples without any enrichment. Additionally, processed and control samples were placed into a 49°C (120°F) incubator for 4 weeks and sampled weekly to estimate shelf life.

Microbiological analysis was conducted on all samples. The samples were transferred to a sterile stainless steel blender jar and ground up without the addition of liquid, 10-g portions were weighed out from each blended sample into a sterile stomacher homogenizer bag followed by the addition of 90 ml of Butterfield's phosphate buffer, pH 7.2. Each bag was then homogenized in a Seward Stomacher (Stomacher Lab Blender Model 400, Seward Medical Limited, London, UK) for 2 min on medium speed. The microbiological analysis of population enumeration was determined via spread plating of 100 µL onto MRS agar LA bacteria (LAB). Total aerobic plate count (APC), *E. coli*/coliform count, and yeast and mold counts were determined on PetriFilm® (3 M). Plates were incubated anaerobically, and PetriFilm® was aerobically incubated for 48 h before colony enumeration. Significant reductions of 4.86 Log cfu/g and 3.98 Log cfu/g in the LAB and APC (respectively) were measured in the hard salami that was processed with SCCO2 for 30 min at 1436 psi and 35°C.

4.2.2 Microbiological Analysis Results

The mashed potatoes and applesauce were not able to be assayed because they did not survive the storage at 49°C (120°F). Contamination occurred at NovaSterilis, and the samples puffed up and burst in storage. It should be noted that this only occurred with the control samples and not the processed samples (Figure 13). The mashed potatoes pictured at top were not processed and puffed during storage until bursting. The processed potatoes (bottom) did not puff from off-gassing and survived the storage duration.

Figure 13: Mashed Potatoes after Storage at 49°C. Top: Unprocessed; Bottom: Processed with Nova 2200 at 1436 psi and 35°C for 10 min (left) and 30 min (right).

5.0 Mechanism of SCCO2 Processing

This chapter discusses research with diffusivity studies, phenolic activity, and confocal laser scanning microscopy initiated at the end of this project in an effort to elucidate the SCCO2 processing mechanism, as previous studies have shown there may be a number of mechanisms involved. One recent study indicates that microbial inactivation is due to SCCO2's powerful solvency that disrupts the membrane lipid structure (Yuk 2009). Another study theorizes that the SCCO2 lowers the pH in the internal part of the cell, causing the destruction of the cell (Damar 2006). It should be noted that SCCO2 is a powerful extraction solvent, and has been used to extract everything from caffeine to flavonoid compounds from foods. With that in mind, one theory suggests that extractions of components from the cell cause the cell death. The SCCO2 permeates through the cell membrane and physically extracts these components out of the cell during the pressure release at the end of the processing (Lin 1992).

5.1 Diffusivity Research

The diffusivity of the SCCO2 into semi-solid and solid foods had not been researched. In order to further understand the diffusing power of the SCCO2, polypropelene Falcon tubes were filled with different concentrations of agar (0.25-1.0%) and 2% of VWR Universal color indicator. The tubes were processed for various times and at various pressures, and the change in color was recorded with cameras and rulers. Figure 14 compares the diffusivity of SCCO2 with 10 min of processing at 4350 psi and with 60 min of processing at the same psi, as illustrated in Agar and pH color indicator filled tubes. When processed for only 10 min, the SCCO2 initially diffused only a very small amount, but then fully diffused throughout the tube 4 h after the processing was stopped. However when processing was continued for 60 min, the SCCO2 diffused throughout the entire tube, and a complete color change occurred regardless of the agar concentration.

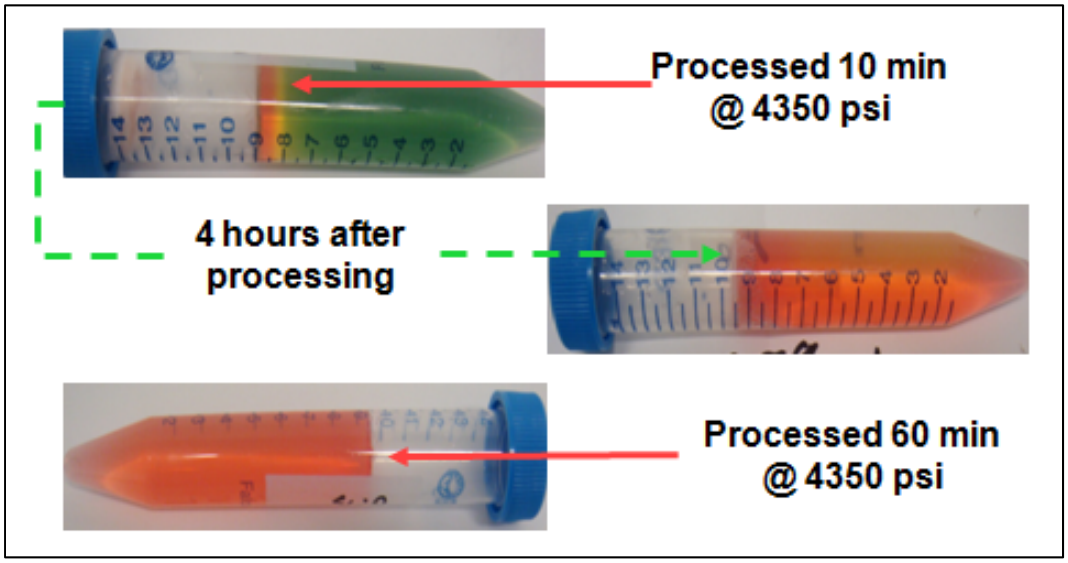

Figure 14: Diffusion of SCCO2 in Agar and pH Color Indicator Filled Tubes when Processed at 4350 psi for 10 min and for 60 min.

5.2 Phenolic Activity

Phenolic compounds are naturally found in the foods that were chosen for the enzymatic inactivations study (apples, potatoes, avocadoes, and lettuce). Each will vary in its content and structure of individual phenolic makeup and has been linked back to its antioxidant activity. In determining the effect of SCCO2 on foods, phenolic content was measured before and after processing.

5.2.1 Phenolic Assay Methodology

Standards for gallic acid, a phenolic compound, were prepared in microcentrifuge tubes in increasing concentrations of 0, 10, 20, 50, and 100 ppm of gallic acid, as shown in Table 4.

Table 4: Standards Prepared for Phenolic Activity Assay

Standard	Gallic Acid Concentration				
	0 ppm	10 ppm	20 ppm	50 ppm	100 ppm
100 ppm gallic acid (µL)	0	10	20	50	100
80% methanol (µL)	100	90	80	50	0
1/30X FCR reagent* (µL)	500	500	500	500	500
Sodium carbonate (µL)	400	400	400	400	400
Total volume (ml)	1.0	1.0	1.0	1.0	1.0

***Folin-Ciocalteu reagent

The standards in Table 4 were added to microcentrifuge tubes in the following order: gallic acid, methanol, FCR reagent (pause for 2 min for reaction), and sodium carbonate. The microcentrifuge tubes were vortexed for 20 s and then stored in the dark for 30 min. While the standards were in the dark, the samples were prepared as shown in Table 5

Table 5: Samples Prepared for Phenolic Activity Assay

Sample	Volume
Sample extract in 100% methanol	100 µL
1/30 FCR	500 µL
Sodium carbonate	400 µL
Total volume	1000 µL (1.0 ml)

The samples were prepared in a very specific order: sample of vegetable/fruit extract, 1/30 FCR (pause for 2 min), and sodium carbonate. They were vortexed for 20 s and stored in the dark for 30 min. The samples were then centrifuged at 10,000 rpm for 1 min and incubated in the dark for 1 h before they were read on a Varian Cary 50 bio spectrophotometer at 765 nm.

5.2.2 Phenolic Assay Results

The processed samples (at 4350 psi for 1 h) and the control samples had equivalent phenolic activity. Therefore, the phenolic activity was not changed due to processing.

5.3 Confocal Imaging

Confocal microscopy was applied to the processed samples in an effort to determine the mechanism of the SCCO2 and its effect on the sample's cellular composition. A Zeiss LSM 710 was used to capture the confocal images. Samples of avocado were soaked in acridine orange and imaged while wet with a 20X objective. Figure 14 shows an explicit difference between the image of the processed sample (at 4350 psi and 40˚C for 60 min) and the image of the unprocessed sample, but further research is needed to determine the significance of these differences.

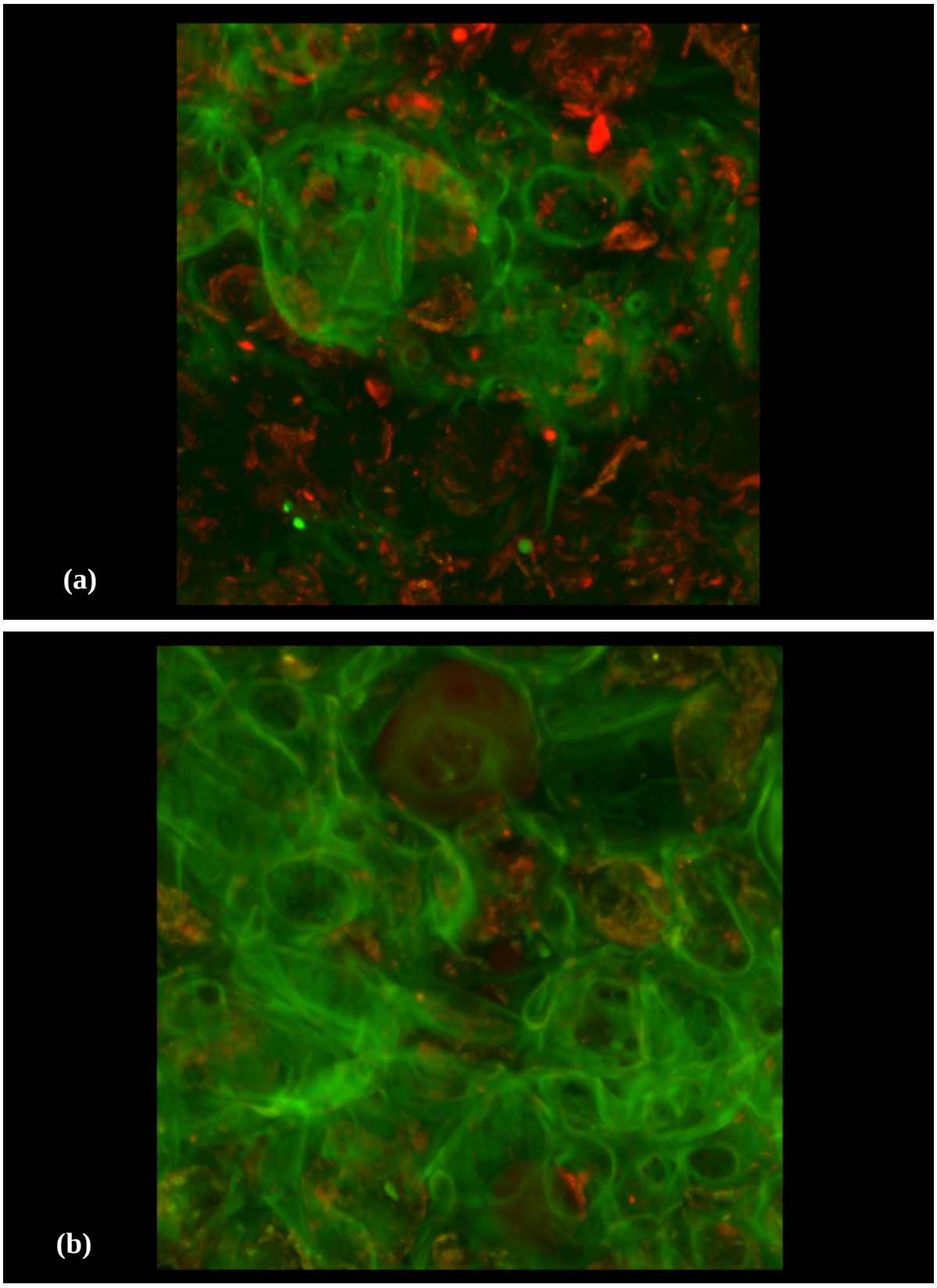

Figure 15. Confocal Laser Images of an Avocado.
(a) Processed with SCCO2 for 45 min at 3000 psi and 40˚C; (b) Unprocessed.

6.0 Conclusions and Future Research Direction

Novel treatments to improve food safety and quality have always been a focus for combat rations. In this study, SCCO2 processing was shown to inactivate microbes and enzymes on basic food models and more complex food matrices and was effective in inhibiting non-enzymatic browning in food samples with minimal impact on food quality. This treatment was successfully used in a broad range of food matrices without the destructive side effects that normally occur through the use of thermal processing.

Although the initial instrumentation (SFT100) was limiting, the results were conclusive and offered a solid foundation for further research on SCCO2 processing. Further testing was conducted using the Nova 2200, a batch system built and designed by the small company NovaSterilis (Ithaca,NY) for SCCO2 sterilization of delicate materials such as live dermal tissue similar in nature to the delicate tissue of fresh fruit. A day's testing in the NovaSterilis lab and a subsequent storage study at NSRDEC proved that the instrument will assist NSRDEC CFD in furthering research in this area.

Research with diffusivity studies, phenolic activity, and confocal laser scanning microscopy was initiated at the end of this project to determine the SCCO2 processing mechanism, which is probably a combination of inactivation mechanisms. The research team intends to extend this research on the mechanism(s) behind the processing in the planned JSN that this has transitioned to JSN 11-05.

The future application of SCCO2 processing may be in coupling it with an existing processing such as high pressure to completely kill spores. Two major industrial gas companies, Praxair and Air Liquide, have explored applications of SCCO2 to liquid foods in pilot scale systems; one such system was used at the University of Florida by Dr. M. Balaban and coworkers. One small company called PoroCrit had a novel membrane-based delivery system for juice processing, but went out of business with the death of the founder. The batch SCCO2 systems recently built by NovaSterilis for use in sterilizing transplant tissues and medical implants proved useful at the end of this project. With the NSRDEC CFD advances in SCCO2 preservation of solid foods, it is possible that this technology will provide a new, non-thermal treatment/preservation method for application to military combat rations.

This document reports research undertaken at the U.S. Army Natick Soldier Research, Development and Engineering Center, Natick, MA, and has been assigned No. NATICK/TR- 12/021 in a series of reports approved for publication.

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Appendix A

Compilation of SFT100 Processing Results on Enzymes and Microbes

Material	Temperature (°C]	Pressure (psi)	Soaking Time (min)	Exhaust Time (min)	Results: Enzymatic (% inactivation)	Results: Microbial (log reduction)
L. reuteri in sterile phosphate buffer	40	1070	30	30		1
	40	1070	60	30		3
	40	1070	120	30		6
	40	3620	30	30		1
	40	3620	60	30		6
	40	3620	120	30		6
	40	↑ 4350	30	30		2
	40	4350	60	30		7
	40	4350	120	30		7
PPO (extracted from potatoes, lettuce, and apples)	40	4350	60	30	100	
	40	4350	30	30	100	
	42	3620	20	20	100	
	40	1000	30	30	49	
	40	2500	30	30	67	
	40	3500	30	30	62	
	40	4350	30	30	100	
	40	3000	60	30	91	
	40	3000	45	30	90	
	40	3000	30	30	91	
	40	3000	15	30	70	
	40	3000	1	30	49	
	40	3000	5	30	47	
Cubes of beef inoculated with *E. coli* 15997	40	1060	60	30		0.8
	40	4350	30	30		1.5
	40	4350	60	30		2.6
Cubes of beef inoculated with *E.coli* 0157:H7	42	4350	10	30		1
	42	4350	20	30		2
	42	4350	30	30		2.1
	42	4350	60	30		2.5
	38	4350	30	30		2
	38	4350	60	30		2.5
	38	4350	120	30		7